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PHYTOCHROME CONTROL OF OSCILLATING LEVELS OF PHENYLALANINE AMMONIA LYASE IN *HORDEUM VULGARE* SHOOTS*

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; phenylalanine ammonia lyase; phytochrome; oscillating enzyme levels; *Avena sativa*; oats.

Abstract—Five-day-old etiolated barley shoots respond to brief illumination with red light by increasing their level of PAL *ca* 50% within 5 hr. When assayed throughout a 26-hr period the photocontrolled PAL shows a pattern of dampened oscillations with peaks at 5, 13 and 22 hr. By the 26th hr after illumination PAL levels return to near the level of the dark control. The amplitude of the red light induction is reversible by far-red light throughout the experimental period as a typical phytochrome response. In contrast, PAL levels in 5-day-old *Avena sativa* shoots do not respond to brief illumination with red light.

INTRODUCTION

PHYTOCHROME control of phenylalanine ammonia lyase (PAL) is well established for many dicotyledonous seedlings,[1,2] yet there seem to be no such reports for monocotyledonous seedlings (Stafford, personal communication). Red far-red photoreversibility studies[3] and action spectra[4] for flavenoids demonstrate phytochrome control of phenolic accumulation in barley. Amrhein and Zenk[5] have shown that 10 hr of white light enhances PAL levels in barley shoots by *ca* 40%.

In an attempt to correlate PAL levels with previous work on photocontrol of barley flavonoids,[3,4] detailed time-course studies were made to characterize the effects of low levels of red (R) and far-red (FR) light on PAL in etiolated barley shoots. *Avena sativa* seedlings were included for comparison in some of the experiments.

RESULTS

Preliminary experiments indicated that continuous R light throughout a 24-hr period led to an increase in PAL above the levels found in the dark controls (Fig. 1). Enzyme activity varied with age and weight of the tissue (see Experimental) and the data in these experiments are reported as per cent change from dark controls grown and harvested with each group of experimental plants. Each datum point was determined in triplicate on at least two different occasions.

* Part III in the series "Phenolic Biosynthesis in Barley Seedlings". For Part II see Ref. 4.

[1] CAMM, E. L. and TOWERS, G. H. N. (1973) *Phytochemistry* **12,** 961.
[2] ZUCKER, M. (1972) *Ann. Rev. Plant Physiol.* **23,** 133.
[3] MCCLURE, J. W. and WILSON, K. G. (1970) *Phytochemistry* **9,** 763.
[4] CARLIN, R. M. and MCCLURE, J. W. (1973) *Phytochemistry* **12,** 1009.
[5] AMRHEIN, N. and ZENK, M. H. (1971) *Z. Pflanzenphysiol.* **64,** 145.

When the amount of R light required to saturate the response was investigated, it was found that 30 sec were sufficient (Table 1). Repeated illumination during the first 5 hr yielded similar results (McClure, unpublished). From this it appears that once set into

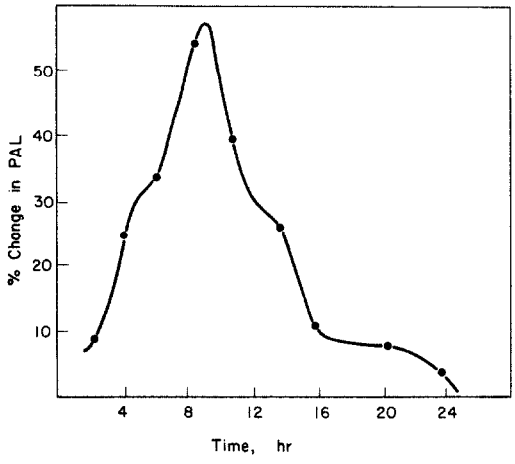

FIG. 1. EFFECTS OF CONTINUOUS R LIGHT ON PAL LEVELS IN PREVIOUSLY ETIOLATED *Hordeum vulgare* SHOOTS.

Five-day-old shoots were placed in continuous R light and harvested at the times indicated. Enzyme levels are plotted to reflect per cent increase from the appropriate dark controls. Data corrected for daily variation and weight of the 4-shoot sample (see Experimental). Red light energy was 1·1 kerg.cm^{-2}.sec^{-1}.

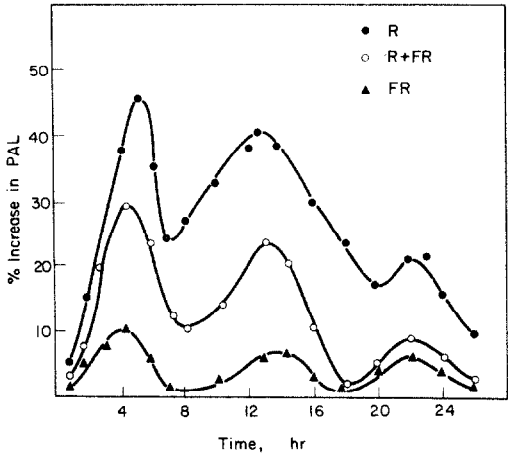

FIG. 2. PAL LEVELS IN 5-DAY-OLD *Hordeum vulgare* SHOOTS IN RESPONSE TO BRIEF ILLUMINATION WITH R, R PLUS FR, OR FR LIGHT.

Previously etiolated plants were given 4 min of R (1·1 kerg.cm^{-2}.sec^{-1}), or 2 min of FR (34 kerg.cm^{-2}.sec^{-1}), or R plus FR light, and returned to the dark until harvested. Enzyme levels plotted to reflect per cent increase from appropriate dark controls. Data corrected for daily variation and weight of the 4-shoot samples (see Experimental).

TABLE 1. PAL LEVELS IN *Hordeum vulgare* SHOOTS GIVEN R LIGHT 5 hr BEFORE HARVEST

Light treatment*	PAL levels	
	Uncorrected enzyme units	Per cent change from dark control†
Dark control	53	Control
30 sec R	80	44
2 min R	60	43
4 min R	93	46
16 min R	59	48
Continuous R	69	46

* Previously etiolated plants were given light on the 5th day after planting and harvested 5 hr later. R light energy was 1·1 kerg.cm^{-2}.sec^{-1}.

† Corrected for daily variation of the dark controls and for fresh weight of the 4-shoot sample (see Experimental).

motion, R light photoincreased PAL in barley is relatively insensitive to further illumination for at least the first 5 hr.

The effects of R, FR, and R plus FR light on PAL levels were then determined at 1- or 2-hr intervals throughout a 26-hr period (the results are shown in Fig. 2). The decrease

in activity after 5 hr was not surprising as this seems to be a frequent characteristic of photocontrolled PAL.[1,2] However, the subsequent increase in activity at 13 hr, without further stimulus, was unexpected.

We considered that the decrease at 7 hr might reflect the intervention of some soluble, low MW, inhibitor that accumulated between the 5th and 7th hr and was removed from the system by the 13th hr. In an attempt to test this we prepared enzyme extracts from dark control plants or from plants given light 5 or 7 hr before harvest. After boiling, these fractions were added to enzyme extracts from plants given light 5 hr earlier. We also prepared methanol soluble extracts from equivalent weights of dark control plants or from plants given light 5 or 7 hr before harvest. The methanol extracts were dried *in vacuo*, taken up in the borate buffer, and added to enzyme extracts of plants given light 5 hr earlier. In no instance did the boiled enzyme preparations have any inhibitory effect. The methanol soluble fractions were all slightly, and equivalently, inhibitory. From this we conclude that the pattern of dampened oscillations is not a simple situation where a soluble inhibitor accumulates in some subcellular compartment, possibly different from the normal subcellular localization of the enzyme, is then extracted with the enzyme preparation and limits the expression of PAL.

To test the photocontrol of PAL in another monocotyledonous plant we examined 4- or 5-day-old seedlings of oats (*Avena sativa*). Red light treatments of from 1 to 30 min had no effect on PAL levels of shoots harvested at 2-hr intervals throughout a 24-hr period. Interestingly, oat shoots contain about 93 enzyme units per g fr. wt whether maintained in the dark or illuminated, while dark-grown barley of a similar age has no more than 65 enzyme units in the dark. The highest levels of PAL that can be unduced in barley by brief R light treatments are approximately equal to those found in dark-grown oats.

DISCUSSION

The R, FR photoreversibility of PAL levels in barley shoots is good evidence for phytochrome control. Additional support comes from the similarity of the various curves (Fig. 2) which may be interpreted as responses to varying levels of P_{fr}.[6] One unusual aspect of the data is that R plus FR is not equivalent to FR alone. However, from Table 1 it is seen that only small amounts of illumination are required to saturate photoincreased PAL in barley shoots. Other work[7] has shown that as little as 1·1 kerg.cm^{-2} of 600 nm light (equivalent to one second of light from the red light source) is highly effective in increasing PAL in this tissue. Thus it is highly likely that the presence of high levels of P_{fr} in the tissues during the 4-min R light treatment (26 kerg.cm^{-2}) preceding the FR light may explain why red plus FR are not equivalent to FR alone.

A lag phase of 1 or more hr is a common feature of phytochrome controlled PAL levels,[1,2] yet lag phases are usually closely related to several factors such as previous illumination and the age of the tissue.[8] In any event, no lag phase can be detected in the light induction of PAL in barley shoots.

The dampened oscillations of PAL in barley shoots in response to light may be explained by one or more of several mechanisms such as allosteric inhibition of the enzyme by the buildup of a pool of reaction products and gradual removal of the product into

[6] Briggs, W. R. and Rice, H. V. (1972) *Ann. Rev. Plant Physiol.* **23**, 293.
[7] McClure, J. W. (1974) *Phytochemistry* **13**, 1071.
[8] Mohr, H. (1972) *Lectures on Photomorphogenesis*, Springer, New York.

other pathways in a cyclic system. However, if this is the case it is not apparent since the addition of boiled enzyme, or methanolic soluble fractions, gave no indication that the soluble constituents differed in their effect on PAL at 5 hr (highest activity) or at 7 hr (reduced activity). Furthermore, the spectrophotometric assay is linear for at least 1 hr with the most active PAL preparations that we examined and shows no signs of production inhibition.

The oscillations may also relate to compartmentalization of enzymes, reflecting PAL synthesis at different rates in different pools. For example, PAL is present in several subcellular compartments as determined by subcellular fractionation studies.[9,10] In barley there appear to be at least two subcellular pools for PAL under different control mechanisms. Isolated etioplast and chloroplast of barley contain PAL but this plastid fraction is not controlled by light[10] while the total PAL of the shoot is photocontrolled (Figs. 1 and 2).

Another system that could explain the oscillations is that of genetic feedback repression where the product of the enzyme reaction inhibits some stage prior to enzyme synthesis. This would allow the product to be shunted into diversionary metabolic pools[1] and would eventually release the enzyme synthesizing system from repression. Such dampened oscillations for enzyme systems have been developed in computer-generated studies of genetic feedback repression[11] and they show all of the salient features of Fig. 2.

From the work of Amrhein and Zenk[5] it is apparent that while aerial tissues of several monocotyledonous and dicotyledonous plants respond to several hours of W light by increasing levels of PAL, striking differences are found between various species. For example, over 200 times more PAL activity is found in the shoots of *Hordeum vulgare* than in the hypocotyl of *Cucumus sativus*. Furthermore, the enhancement of PAL by light varies from a factor of 0·96 (no enhancement) in *Zea mays* epicotyls to a factor of 6·75 in the epicotyl of *Pisum sativum*. If the variation in PAL levels and the degree of response to light found by these workers is representative, then this may explain why photocontrolled PAL may be limiting for further phenolic accumulation in some tissues where it is present in low amounts in the dark and not limiting in others where it is in high levels without the intervention of light. This may be the case in barley (low levels in the dark with photoinduction) and in oats (high levels in the dark, no light effects).

The R, FR photoreversibility studies show that phytochrome controls PAL in barley shoots. Relating this to flavonoid levels in barley, higher levels of R light are required to saturate phytochrome control of flavonoid accumulation than to saturate increased PAL levels. For example, 30 sec of R light will fully saturate the requirements for PAL (Table 1) while 4 min from the same source are required for maximal flavonoid accumulation in the same tissue.[3] That the photoreceptors for both PAL increase and for flavonoid increases in barley are similar in the R regions of the spectrum will be shown in the next paper in this series.[7]

EXPERIMENTAL

Plant material and light treatments. Atlas 46 barley (*Hordeum vulgare* L.), a gift of G. Wiebe, U.S.D.A., Beltsville, Maryland, or Clintford oats (*Avena sativa* L.), from the Farm Bureau Cooperative, Columbus, Ohio, were grown in glass beakers on H_2O-saturated vermiculite in 23° incubators in a darkroom.[3] All manipulations from soaking the seeds through centrifuging the enzyme preparations were carried out in the dark or under a dim

[9] Camm, E. L. and Towers, G. H. N. (1973) *Phytochemistry* **12**, 1575.
[10] Saunders, J. A. and McClure, J. W. (1972) *Am. J. Botany* **59**, 673 (abstract only) and in preparation.
[11] Tiwari, J. and Fraser, A. (1973) *J. Theoretical Biol.* **39**, 679.

green safelight. The R and FR light sources (producing 1·1 and 34 kerg.cm^{-2}.sec^{-1} respectively) have been described.[3]

Enzyme extraction and PAL assay. PAL was assayed by a technique modified from Zucker.[12] Four shoots were excised at the point of emergence from the caryopsis, weighed, and thoroughly ground in a chilled Ten Broeck homogenizer containing 8 ml 0·025 M borate buffer (pH 8·8) with 23 μl of mercaptoethanol and 0·3 g Polyclar AT. The Polyclar AT was soaked overnight in the buffer and mercaptoethanol before use. The homogenate was centrifuged at 4° for 30 min at 30900 *g*. The PAL reaction mixture was 1 ml of the supernatant, 1 ml of 50 μM L-phenylalanine, and 1 ml of 0·025 M borate buffer. Controls contained dist. H_2O in place of L-phenylalanine. The change in absorbtivity at 290 nm was monitored for 1 hr at 40°. To ensure that the change in absorbtivity at 290 nm was a reflection of PAL activity, the reaction mixture from several of the experiments was acidified with HCl, extracted with diethyl ether, dried *in vacuo*, and the residue suspended in buffer for chromatographic and spectrophotometric assay. One enzyme unit is defined as a change in absorbtivity at 290 nm of 0·01/g. fr. wt/min.

Enzyme activity in triplicate dark controls was invariably similar ($\pm$*ca* 5%) but displayed slight daily variation from samples of a similar weight obtained on other occasions. Furthermore, PAL content per g of tissue varies directly with the weight of the 4 plumule sample. For example, dark controls weighing 0·220 g averaged 65 enzyme units while those weighing 0·345 averaged 42 enzyme units. When several hundred dark control values were compared a strictly linear relationship was found between weight and PAL activity. This is most likely a reflection of slight differences in H_2O content of the tissues and these differences may cause significant changes in PAL levels in barley seedlings.[13] Accordingly, PAL activity is corrected for daily variation and weight of the tissues.

Acknowledgements—This work was supported by the National Science Foundation (Grant GB 16636) and by funds from the Miami University Research Committee. The expert technical assistance of Mrs. Marjorie Smith is gratefully acknowledged.

[12] ZUCKER, M. (1968) *Plant Physiol.* **43,** 365.
[13] SAUNDERS, J. A. and MCCLURE, J. W. (1973) *Plant Physiol.* **51,** 407.

Note added in proof: Improved techniques of organelle disruption have recently allowed us to demonstrate phytochrome control of PAL in *Hordeum* plastids